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The NS2 Protein Generated by the Parvovirus Minute Virus of Mice Is Degraded by the Proteasome in a Manner Independent of Ubiquitin Chain Elongation or Activation

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The NS2 protein generated by the parvovirus minute virus of mice is very labile, having a half-life during infection of approximately 90 min. The degradation of NS2 is blocked by proteasome inhibitors but is likely ubiquitin independent: NS2 does not form detectable higher molecular weight ubiquitin-containing conjugates, and NS2 degradation requires neither ubiquitin chain elongation nor intracellular ubiquitin activation. We have also identified a region in the carboxyl half of NS2 that is required for its proteasome-mediated degradation.

INTRODUCTION

The autonomous parvoviruses encode two nonstructural proteins, NS1 and NS2, which play critical roles in the viral life cycle (Vanacker and Rommelaere, 1995). Because the mRNAs that encode these proteins are both generated from the same pre-mRNA molecule, the relative abundances of NS1 and NS2 are controlled posttranscriptionally, at the level of alternative splicing and protein stability (Cotmore and Tattersall, 1990; Schoborg and Pintel, 1991; Pintel *et al.,* 1995). NS1 is a very stable 83-kDa nuclear phosphoprotein (with a half-life of over 6 h in infected cells), encoded by the singly spliced R1 mRNA, that has multiple critical roles during viral replication (Vanacker and Rommelaere, 1995; Cotmore and Tattersall, 1995). NS2, encoded by the doubly spliced R2 mRNA, is a 25-kDa phosphoprotein that is present both in the nucleus and in the cytoplasm of infected cells; the phosphorylated forms are found only in the cytoplasm (Clemens *et al.,* 1990; Cotmore and Tattersall, 1990). NS2 is very labile, yet it accumulates to high levels and is required for productive minute virus of mice (MVM) infection in a host-range-dependent manner (Naeger *et al.,* 1990, 1993; Cotmore and Tattersall, 1990; Li and Rhode, 1991; Cotmore *et al.,* 1997). The role that NS2 plays during infection is not yet clear. NS2-null mutants show a striking defect in the accumulation of all virus replicative forms in restrictive cells, and NS2 mutants have been shown to exhibit defects in capsid assembly (Naeger *et al.,* 1990, 1993; Li and Rhode, 1991; Cotmore *et al.,* 1997). NS2 is known to bind to three cellular proteins, the nuclear export factor Crm1 (Bodendorf *et al.,* 1999; Ohshima *et al.,* 1999) and two of the 14-3-3 family of multifunctional signaling adaptor proteins, ϵ and β or ζ (Brockhaus *et al.,* 1996). The consequence of these interactions are not yet clear.

The degradation of most cellular and many viral proteins is carried out by the 26S proteasome, a cellular protease made up of two distinct multisubunit complexes, the 20S catalytic core and the 19S regulatory cap (see DeMartino and Slaughter, 1999; Zwickl *et al.,* 1999 for review). Although the 20S core proteasome can be made active *in vitro* upon mild denaturation, its activity *in vivo* is thought to require association with the 19S cap complex (DeMartino and Slaughter, 1999). Protein degradation is usually mediated by direct conjugation of multi-ubiquitin chains to degradation substrates, which are then recognized by the 19S regulatory cap, unfolded, and fed into the inner chambers of the 20S core proteasome in an ATP-dependent manner (Hershko and Ciechanover, 1998; Zwickl *et al.,* 1999). There are only a few reported exceptions to this mode of degradation. Ornithine decarboxylase, through interaction with the inhibitor protein antizyme, is directed to the 26S proteasome where it is inactivated, sequestered, and then degraded by the proteasome in an ATP-dependent and ubiquitin-independent manner (Murakami *et al.,* 1992, 1999). The cyclin-dependent kinase, p21, although a substrate for ubiquitination, was also recently shown to be degraded by the proteasome in a ubiquitin-independent manner (Sheaff *et al.,* 2000).

It seems likely that the instability of NS2 is important for its function during virus infection. In this report we show that the degradation of MVM NS2 is performed by the proteasome; however, it is highly likely that NS2 degradation is independent of ubiquitin conjugation. In

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addition, we have identified a region in NS2 that is involved in its proteasome-mediated degradation.

RESULTS

Degradation of NS2 is dependent upon the proteasome

During wild-type viral infection, NS2 is quite labile. Pulse–chase experiments suggested that its half-life, in infected murine A9 cells, was approximately 90 min (Fig. 1). The degradation of NS2 was dramatically blocked by well-characterized inhibitors of the 20S proteasome. Addition to infected cells of MG132 [a proteasome substrate analog which competitively inhibits both the chymotrypsin-like and the peptidylglutamyl-peptide-hydrolyzing (PGPH) activity of the 20S proteasome in addition to other cellular proteases (Rock *et al.,* 1994)], lactacystin [which specifically inhibits the chymotrypsin-like, trypsin-like, and PGPH activity of the 20S proteasome by irreversibly modifying all catalytic subunits (Fenteany *et al.,* 1995)], or epoxomicin [which strongly and very specifically inhibits the chymotrypsin-like and, to a lesser extent, the trypsin-like and PGPH activity of the 20S proteasome by specifically and irreversibly binding covalently to the LMP7, MECL, X, and Z catalytic subunits (Meng *et al.,* 1999)] increased the half-life of NS2 significantly. At 90 min, when approximately half of the untreated NS2 samples had degraded, levels of the inhibitor-treated samples remained essentially unchanged. Extrapolation of the degradation curves in Fig. 1 showed that MG132, lactacystin, and epoxomicin increased the half-life of NS2 by approximately eightfold, by approximately sixfold, and to almost complete stability for the duration of the assay (180 min), respectively (Fig. 1). These results indicated that the 20S proteasome mediates NS2 degradation. NS2 generated from transfected plasmids exhibits a lability and sensitivity to proteasome inhibitors similar to that of NS2 generated during infection.

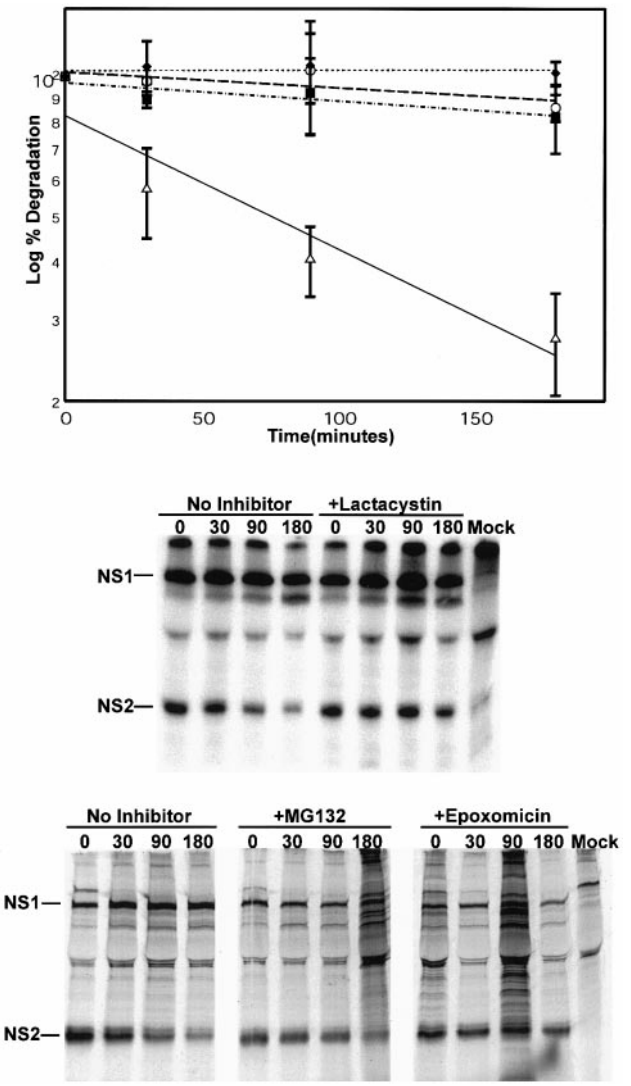

FIG. 1. Degradation of NS2 in the presence of proteasome inhibitors. Pulse–chase experiments were performed, using α-NS1/2 N-terminal antibody, either in the absence or in the presence of proteasome inhibitor lactacystin (5 μM), MG132 (10 μM), or epoxomicin (10 μM), utilizing chase periods of 0, 30, 90, or 180 min following MVM infection of A9 cells, as shown. Representative experiments are shown at the center and bottom and NS1 and NS2 are designated. A graph showing the average of three experiments as measured by phosphorimager analysis in the absence (—, △) and presence of lactacystin (– · –, ■), MG132 (– – –, ○), and epoxomicin (- - -, ◆) is shown at the top. Best fit linear regression lines are shown and indicate a half-life for NS2 of approximately 90 min. Relative phosphoimager units of NS2 were standardized to relative units of stable NS1 within each sample to control for loading errors.

NS2 does not form detectable higher molecular weight ubiquitin-containing conjugates in the presence of a proteasome inhibitor

The great majority of proteins that are degraded by the 26S proteasome are targeted for degradation by ubiquitin conjugation (Hershko and Ciechanover, 1998; Zwickl *et al.,* 1999). Such conjugates are typically detectable in the presence of proteasome inhibitors (Clurman *et al.,* 1996). We therefore investigated whether NS2 formed ubiquitin conjugates in the presence of MG132. Cyclin E, known to be degraded by the 26S proteasome in a ubiquitin-directed manner, served as a positive control. Following cotransfection of A9 cells with both a HA-tagged ubiquitin and a myc-tagged cyclin E-expressing plasmid, and the subsequent addition of MG132, HA–ubiquitin-containing, higher molecular weight conjugates of specifically immunoprecipitated cyclin E could be readily demonstrated by Western analysis using α-HA antibody (Fig. 2, bottom left). α-HA antibody also specifically immunoprecipitated material subsequently recognized on Western by α-c-myc antibody (Fig. 2, bottom center). Similar experiments clearly demonstrate ubiquitin conjugation to p21 (data not shown). In contrast,

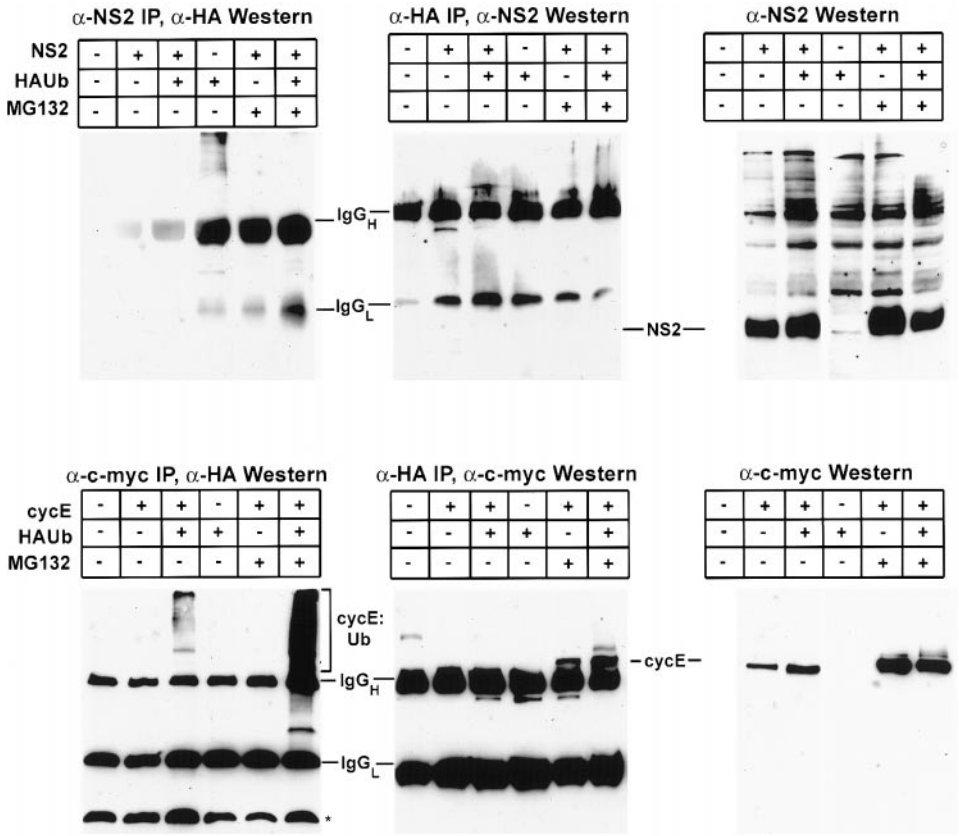

FIG. 2. There are no detectable higher molecular weight ubiquitin-containing NS2 conjugates in the presence of the proteasome inhibitor MG132. A92L cells were transfected with pCMVNS2 (top) or pCS2-MTcyclinE (bottom). pHAUb was cotransfected with the test plasmids where indicated. MG132 was added to cells 6 h posttransfection where indicated. 18 h posttransfection cells were split into three samples and immunoprecipitated with either α-NS2 (top left) or α-c-myc (to precipitate myc-tagged cyclin E, bottom left) and loaded on SDS–PAGE followed by Western blot analysis with α-HA antibody, immunoprecipitated with α-HA antibody followed by Western blot analysis with either α-NS2 (top middle) or α-c-myc (bottom middle) antibody, or loaded directly on SDS–PAGE without immunoprecipitation followed by Western blot analysis with α-NS2 (top right) or α-c-myc (bottom right) antibody. The identity of the band marked by an asterisk is unknown. NS2, cyclin E, and higher molecular weight ubiquitin-containing forms of cyclin E are identified. Cross-reactive IgG heavy and light chains are also identified; however, these are underrepresented in the particular gel shown in the upper left.

under similar experimental protocols done in parallel, such higher molecular weight, HA–ubiquitin-containing conjugates of NS2 were not detected (Fig. 2, top left and center), although NS2 was expressed at high levels (Fig. 2, top right) and was readily precipitated with the same α-NS2 antibody from labeled cells transfected in parallel (data not shown). Similar experiments also repeatedly failed to detect NS2 conjugates with endogenous ubiquitin in infected or transfected cells (data not shown). In addition, the conjugation of ubiquitin to NS2 could not be detected by pull-down assays using either labeled GST–ubiquitin mixed with cold *in vitro*-generated NS2 or cold GST–ubiquitin mixed with labeled *in vitro*-generated NS2 (data not shown). These results, although negative in nature, suggested that perhaps degradation of NS2 by the 20S proteasome did not require ubiquitination of NS2. We therefore chose to further examine NS2 degradation following disruption of ubiquitin chain elongation and ubiquitin activation, two critical steps required for ubiquitin-dependent degradation, as has been done previously to establish that the degradation of p21 was ubiquitin independent.

NS2 degradation does not require ubiquitin chain elongation

Processive ubiquitin chain elongation, a required step for recognition of ubiquitin-mediated degradation substrates by the regulatory subunit of the 26S proteasome, can be blocked by the lysine-less dominant-negative form of ubiquitin, UbR7, and transfection of a UbR7-expressing plasmid blocks the degradation of proteins that are targeted to the proteasome by ubiquitin (Sheaff *et al.,* 2000). While transfection of a UbR7-expressing plasmid clearly blocked the degradation of cotransfected cyclin E (Fig. 3, top), it had no significant effect on the lability of cotransfected NS2 (Fig. 3, center). The stability of p21, a protein known to be ubiquitinated, but whose degradation does not require ubiquitination (Sheaff *et al.,* 2000), was similarly unaffected by the dominant-negative

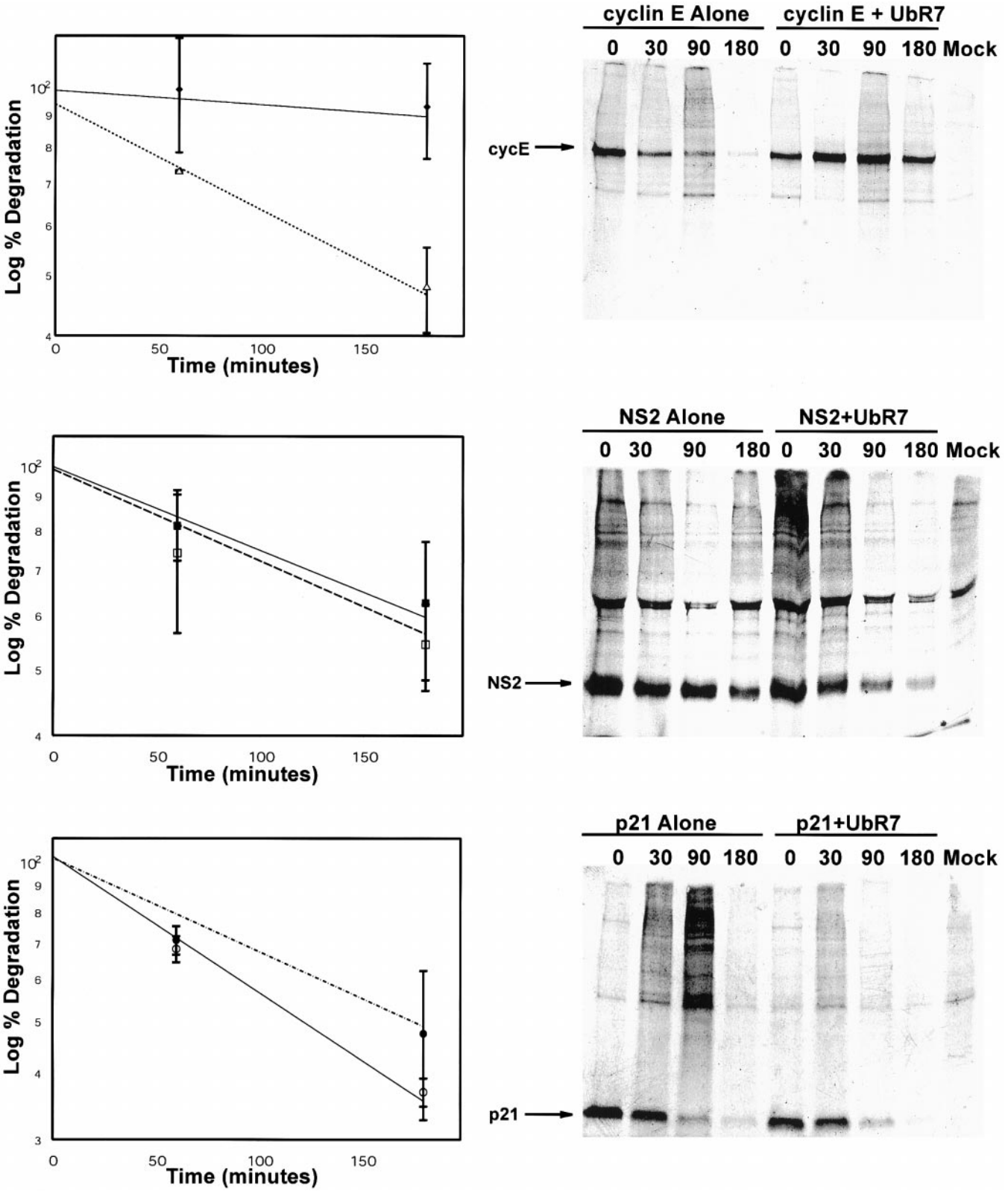

FIG. 3. NS2 degrades normally in the absence of ubiquitin chain elongation. A92L cells were transfected with pCS2-MTcyclinE (top), pCMVNS2 (middle), or pCS2p21 (bottom) either alone or cotransfected with pUbR7. 18 h posttransfection, cells were pulse labeled with ^{35}S and then chased for 0, 30, 90, and 180 min. Immunoprecipitations were done with α-c-myc to detect cyclin E (top), α-NS2 (middle), or α-p21 (bottom) antibodies. A representative set of experiments is shown on the right. Graphs depicting the log % degradation of cyclin E in the absence (- - -, △) and presence (—, ◆) of UbR7 (top left), NS2 in the absence (– – –, □) and presence (—, ■) of UbR7 (middle left), and p21 in the absence (—, ○) and presence (– • –, ●) of UbR7 (bottom left) are shown and represent the average of three separate experiments as measured by phosphorimager analysis. The best fit linear regression line is shown for each. The positions of NS2, p21, and cyclin E (cycE) are shown.

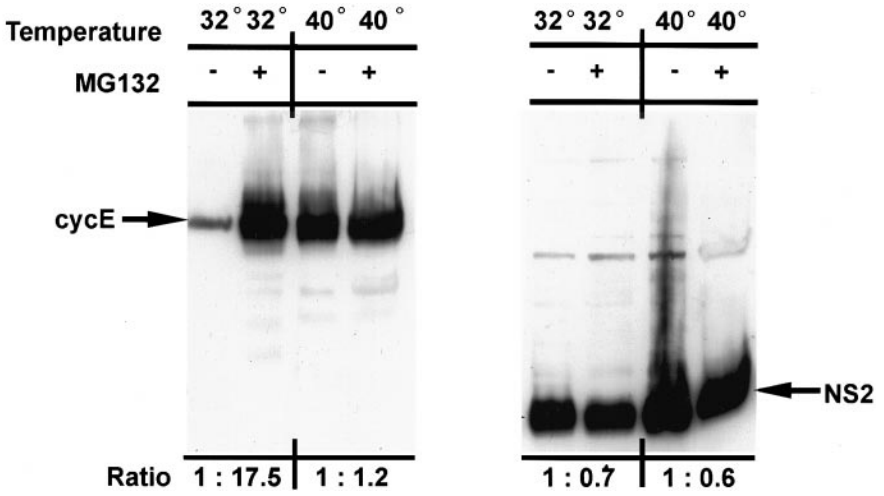

FIG. 4. The accumulation of NS2 is not affected by the absence of intracellular ubiquitin activation. TsA1S9 cells were transfected with pCST-MTcyclinE or pCMVNS2 at the permissive temperature of 32°C. Six hours posttransfection, where indicated, cells were shifted to the restrictive temperature of 40°C and MG132 (10 μM) was added. 18 h posttransfection cells were collected and equivalent amounts of protein were run on SDS–PAGE. Western blot analysis was performed using either α-c-myc to detect cyclin E or α-NS2 as indicated. Blots were exposed to a chemiluminescence screen and phosphorimager units were determined. A representative experiment is shown, and the ratios given at the bottom are the average protein accumulations measured in the absence and presence of MG132, at both 32 and 40°C (permissive and restrictive for cellular E1, respectively), from two experiments. The positions of NS2 and cyclin E (cycE) are shown.

mutant of ubiquitin (Fig. 3, bottom). These results are consistent with the model that NS2 degradation does not require prior multiubiquitination.

The accumulation of NS2 is not affected by the absence of intracellular ubiquitin activation

The activation of ubiquitin, which is catalyzed in an ATP-dependent reaction by the cellular E1 enzyme, is a necessary step in ubiquitin-dependent degradation (Hershko and Ciechanover, 1998). In mammalian cells there is a single E1 protein, and thermal inactivation of temperature-sensitive mutants of this protein results in the stabilization of protein targets that require ubiquitination for degradation (Cox *et al.,* 1995). The murine L cell-derived tsA1S9 and other cell lines (Cox *et al.,* 1995; Clurman *et al.,* 1996) which contain a mutation in the E1 gene rendering its product inactive at 40°C have been used to implicate ubiquitination in the degradation process of certain cellular proteins, like cyclin E (Clurman *et al.,* 1996), in the following manner. Following transfection and expression at the permissive temperature of 32°C, at which the E1 protein is active, the ubiquitin-dependent cyclin E protein accumulated to a much greater degree in the presence of the proteasome inhibitor MG132 than it did in its absence (Fig. 4, left). At 40°C, however, at which E1 was thermally inactivated and hence ubiquitin was not activated, there was little difference in the accumulation of cyclin E in the presence vs the absence of inhibitor (Fig. 4, left). In parallel experiments, however, the ratio of the accumulation of NS2 in the presence vs the absence of inhibitor following transfection of tsA1S9 cells was found to be similar at temperatures permissive, and restrictive, for E1 activity (Fig. 4, right). These results further suggested that degradation of NS2 is not a ubiquitin-mediated process.

Although we have determined by pulse–chase experiments that the half-life of NS2 is increased in the presence of MG132, we also observed, for reasons as yet unclear, a general decrease in the overall accumulated levels of NS2 in the presence of proteasome inhibitors. This observation, together with the fact that cyclin E is three- to fourfold more labile than NS2 (Fig. 3, top), can explain why cyclin E is seen to accumulate to a greater extent during the 12-h inhibitor treatment at the permissive temperature than does NS2 (Fig. 4).

Mapping the region of NS2 that governs degradation

The *cis*-acting signals that target proteins for degradation via the ubiquitin cascade have been extensively studied (Hershko and Ciechanover, 1998); however, little is known about the *cis*-acting signals that might target proteins for degradation in a ubiquitin-independent manner. We next, therefore, attempted to identify a region, or regions, of NS2 that might govern its lability. We found that for NS2, this signal is complex.

Because NS1 and NS2 share 84 amino acids at their amino terminus, and NS1 is a very stable protein, we suspected that if NS2 had a signal that governed its lability, at least a part of this signal might be in the carboxyl half of the molecule. To investigate this region further, we initially examined NS2 molecules generated by a mutant, which has been previously described (Naeger *et al.,* 1990), in which a translation termination signal was introduced into the infectious clone of MVM imme-

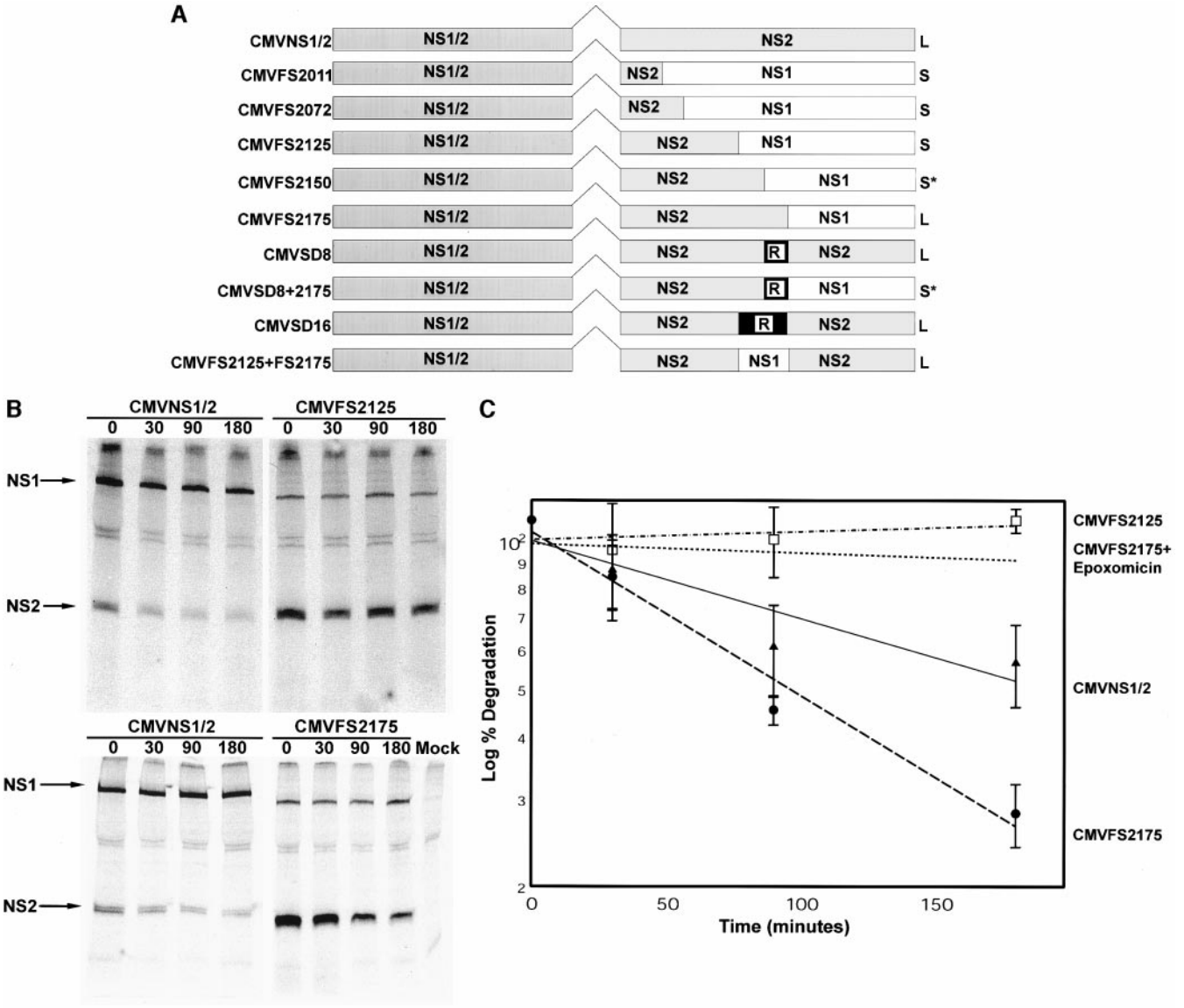

FIG. 5. The NS2 C-terminus is required for its proteasome-mediated degradation. (A) The mutations described and their relative labilities shown in graphical form. S, stable; L, labile; R, random sequence; * indicates mutants that were more stable than wild-type NS2 but not as stable as FS2125. (B) Representative experiment showing the degradation of NS2 generated by CMVFS2125, CMVFS2175, and CMVNS1/2. A92L cells were transfected with NS2 wild-type and mutant expression plasmids as indicated. 18 h posttransfection, cells were pulse labeled for 1 h with ^{35}S and then chased for 0, 30, 90, and 180 min. Immunoprecipitation was performed using an α-NS1/2 N-terminal antibody. NS1 and NS2 are indicated. (C) The averages of five, seven, and nine experiments with CMVFS2125, CMVFS2175, and CMVNS1/2, respectively, as determined by phosphorimager analysis. NS2 counts were equilibrated to NS1 counts to control for sample loading error. Also shown in C is the degradation of pCMVFS2175-generated NS2 in the presence of 10 μM epoxomicin. The best fit linear regression lines for each are plotted.

diately upstream of the small intron at nt 2268. The NS2 molecules produced by this mutant had a lability indistinguishable from that of wild type (data not shown), indicating that amino acid heterogeneity at the very carboxyl terminus of NS2 was likely not involved in NS2 degradation and suggesting further that the labilities of all the individual isoforms of NS2 were regulated similarly.

We next examined a series of frame-shift mutations between ORF 2 and ORF 3 which replaced residues of NS2 with those of NS1 at various positions (Fig. 5). When the NS2 reading frame was shifted such that NS1 sequences replaced NS2 sequences downstream of nt 2011 (CMVFS2011, Fig. 5A), nt 2072 (CMVFS2072, Fig. 5A), or nt 2125 (CMVFS2125, Figs. 5A–5C), the resulting molecule was stabilized >10-fold, suggesting that a region required for lability lay between nt 2125 and nt 2268. When a frame shift was introduced at nt 2175, so that NS2 sequences were retained until this point (CMVFS2175), the resultant molecule regained instability of a magnitude at least as great as that of wild-type NS2 (Figs. 5A–5C). The degradation of CM-

VFS2175-generated NS2, like that of wild-type NS2, was also clearly mediated by the proteasome: treatment with epoxomicin stabilized this mutant protein 10-fold (Fig. 5C). Molecules in which a frame shift was introduced at nt 2150 showed an intermediate phenotype (CMVFS2150, Fig. 5A). These results suggested that aa 131–146 in the region of NS2 between nt 2125 and nt 2175 were involved in its degradation by the proteasome; however, it remained possible that the addition of the NS1 amino acids that correspond to the nt 2125–2175 region conferred stability to the CMVFS2125-generated NS2 chimera. Replacing the NS1 sequence with random amino acid sequence between nt 2150 and nt 2175 in the CMVFS2175 frameshift mutant generated a molecule more stable than wild-type (CMVSD8+2175, Fig. 5A); furthermore, when amino acids 131–146 of otherwise wild-type NS2 were replaced with NS1 amino acids, the resulting protein was unstable. These results argued against the possibility that the NS1 residues provided a stabilizing element (CMVFS2125+FS2175, Fig. 5A). However, when we then replaced the residues between nt 2150 and nt 2175 or nt 2125 and nt 2175 of the wild-type NS2 molecule with a random sequence to determine if this region was sufficient for NS2 lability (CMVSD8 and CMVSD16, respectively), the resultant molecule remained labile (Fig. 5A), suggesting that although amino acids 131–146 are involved in the proteasome-mediated degradation of NS2, this region is not the sole determinant of NS2 lability.

DISCUSSION

The degradation of NS2 is highly sensitive to three proteasome inhibitors of different specificity; however, we were unable to detect conjugation of ubiquitin to NS2 in any of the assays that we performed, either *in vivo* or *in vitro,* although ubiquitin conjugation to both cyclin E and p21 was clearly demonstratable in our assays. These results, although negative, suggested that ubiquitin conjugation to NS2 may not be necessary for its degradation. As done to establish another example of ubiquitin-independent degradation (Sheaff *et al.,* 2000), we further examined NS2 degradation following the disruption of two critical steps required for ubiquitin-dependent degradation, ubiquitin chain elongation and ubiquitin activation. Blocking ubiquitin chain elongation using the dominant-negative UbR7 form of ubiquitin efficiently blocked cyclin E degradation, but had little effect on the half-life of NS2 (or p21). Inhibiting the ubiquitin-dependent degradation pathway even earlier, by blocking the activation of ubiquitin in E1 temperature-sensitive cell lines, again altered the stability of the ubiquitin-dependent cyclin E, but had little to no effect on the stability of NS2. Taken together, these results led us to conclude that NS2 degradation, although proteasome dependent, is highly likely to be independent of ubiquitination.

The mechanism of degradation of proteins that are degraded by the 26S proteasome in a ubiquitination-independent manner is not well understood. Ornithine decarboxylase, the best understood ubiquitin-independent degradation substrate, must interact with the inhibitor protein antizyme to be degraded by the 26S proteasome (Murakami *et al.,* 1992), and it has been proposed that this interaction may induce a conformational change and expose a C-terminal degradation signal of ODC, which is then targeted to the interior of the proteasome (Murakami *et al.,* 1999). Although NS2 has been shown to interact with three cellular proteins [Crm1 (Bodendorf *et al.,* 1999; Ohshima *et al.,* 1999) and two 14-3-3 family members, ϵ and β or ζ (Brockhaus *et al.,* 1996)], and the region of NS2 that interacts with the 14-3-3 proteins (N. Salome, personal communication) is just downstream of a region of NS2 involved in its lability, there is no evidence yet that these proteins play a role in NS2 degradation. When the interactions between NS2 and these proteins were disrupted by mutation, degradation of NS2, as measured by pulse–chase assay of bulk NS2, was relatively unaffected (C. L. Miller and D. J. Pintel, unpublished).

Viral infection can lead to apoptosis of infected cells, and the NS1 protein of parvoviruses has been shown to induce apoptosis in cells when ectopically expressed (Moffatt *et al.,* 1998). This raised the possibility that NS2 degradation may have been mediated by activation of the cellular proteases that are responsible for the bulk of apoptotic protein cleavage. Treatment of CMVNS1/2-transfected cells transfected with the general caspase substrate inhibitor z-vad-FMK (Luo *et al.,* 1998), however, did not result in the stabilization of NS2. It also seems unlikely that NS2 is first cleaved by other cellular proteases and then degraded by the proteasome, because proteasome inhibition resulted in the stabilization of full-size NS2 molecules (as determined by SDS–PAGE, see Fig. 1). It is conceivable that NS2 is recognized and binds directly to the 26S proteasome where it is then degraded. Although there have been reports of the proteasome being capable of degrading unfolded substrates *in vivo* (DeMartino and Slaughter, 1999), there is little reason to believe that NS2 is present in an unstructured form during MVM infection. The selective degradation of proteins is often part of a regulatory mechanism involving timing control; however, NS2 accumulates to high levels throughout MVM infection (Cotmore and Tattersall, 1990; Schoborg and Pintel, 1991). It is intriguing that NS2 would be made at such high levels only to be then degraded very quickly. This would be compatible with a function of NS2 resulting in its degradation, such that new protein is always needed.

There has been considerable attention given to the identification of *cis*-acting sequences that target proteins

for ubiquitination (Hershko and Ciechanover, 1998). Very little is known, however, about the regions that may target proteins for degradation in a ubiquitin-independent manner. Our attempts to identify a region of NS2 that governs its instability were only partially successful. Replacement of NS2 sequences with those of NS1 suggested that residues between nt 2125 and nt 2175 were important for the proteasome-mediated degradation of NS2; however, these residues were not the only region of NS2 that could lead to its destruction because replacing them with random sequence did not result in a stable NS2 molecule. In fact, any mutant in which the region between either 2125 and 2175 *or* 2175 and 2268 contained wild-type NS2 sequences generated an unstable NS2 protein. Those mutants in which both the regions between 2125 and 2175 *and* 2175 and 2268 were either altered or changed completely generated an NS2 protein that was more stable than wild type. Whether these regions participate in affecting the stability of NS2 directly or have other effects (i.e., on localization or phosphorylation) that might affect the stability of NS2 indirectly is not known. These results do suggest, however, that NS2 contains at least two distinct *cis*-acting elements involved in its degradation, either of which is sufficient without the other. Further studies are under way to identify these elements more precisely. A frameshift mutation that replaced the carboxyl terminus of NS1 with that of NS2 (CMVFSX27) was as stable as wild-type NS1, suggesting that these signals are not sufficient to confer lability either in general or, more specifically, to the rather large and stable, nuclearly localized, NS1.

MATERIALS AND METHODS

Cells, viruses, and transfections

Murine A92L cells were maintained and passaged as described (Tattersall and Bratton, 1983). The murine L-cell derivative tsA1S9 (Cox *et al.,* 1995) was maintained at 32°C. Wild-type and mutant MVMp virus was grown and titered on 324K cell monolayers and used to infect A92L cells at a multiplicity of 3. NS2-2268 has been previously described (Naeger *et al.,* 1990). The MVM mutant which loses interaction with 14-3-3 proteins as a result of changing NS2 aa 149 from T to A was made as described (N. Salome, personal communication; Brockhaus *et al.,* 1996). The NS2:Crm1 interaction mutation (which was made according to N. Salome, personal communication, and Ohshima *et al.,* 1999), changed MVM nt 1990–2008 such that the NS2 amino acids change from aa 84–FGTLJI–aa 89 to aa 84–VCPVAV–aa 89, without altering the NS1 amino acid sequence. Transient transfection assays were performed with Lipofectamine and Plus reagent as recommended by supplier (Gibco BRL, Grand Island, NY). Cells were transfected with 2 μg plasmid DNA, when a single plasmid was transfected, or 1 μg each plasmid DNA when cotransfected.

Plasmid constructs

The pCMVNS1/2 plasmid was made by inserting MVM sequences 225–4342 (missing nt 2652–3996) into the MCS of pcDNA 3.1 (Invitrogen) such that both full-length NS1 and NS2 are expressed. pCMVNS2 was constructed by replacing the *Eco*RV–*Xho*I fragment of pCMVNS1/2 with the same fragment from the NS2 cDNA clone (Clemens *et al.,* 1990) so that only NS2 was expressed. The CMVFS2125, CMVFS2150, and CMVFS2175 mutants were constructed by overlap PCR mutagenesis (Haut and Pintel, 1998), such that a single nucleotide was removed at nt 2125, 2150, and 2175 respectively, shifting the NS2 ORF 2 into the NS1 ORF 3 at that point. To generate pCMVFS2011, nt 1248–4342 (minus 2652–3996) of pfs2011 (Gersappe and Pintel, 1999), an MVM plasmid that has a single nucleotide deletion at nt 2011, shifting the NS2 ORF 2 into the NS1 ORF 3 at that point, was inserted into pCMVNS1/2. To generate pCMVFS2072, nt 1248–4342 (minus 2652–3996) of Tb60 (Naeger *et al.,* 1992), a plasmid that has a single nucleotide deletion at nt 2072, shifting the NS2 ORF 2 into the NS1 ORF 3 at this point, was inserted into pCMVNS1/2. To generate pCMVX27, nt 1248–4342 (minus 2652–3996) of X27 (Naeger *et al.,* 1992), a plasmid that has a two-nucleotide deletion at nt 2072, shifting the NS1 ORF 3 into the NS2 ORF 2 at that point, was inserted into pCMVNS1/2. pCMVSD8 was generated by overlap PCR mutagenesis such that eight random amino acids replaced NS2 residues 131–139 of pCMVNS1/2, changing aa 139–SWFQSLPR–aa 146 to aa 139–GGVKGYAG–aa 146. pCMVSD16 was made by overlap PCR mutagenesis such that 16 random amino acids replaced NS2 residues 131–146 in pCMVNS1/2, changing NS2 from aa 131–CRNPEHWGSWFQSLPR–aa 146 to aa 131–GGDAQYRRGGVKGVAG–aa 146 without altering the NS1 protein. pSD8+2175 was also made by overlap PCR mutagenesis such that eight random amino acids replaced residues 139–146 in pCMV2175, changing aa 139–SWFQSLPR–aa 146 to aa 139–GGVKGVAG–aa 146. pCMVFS2125+FS2175 was made by overlap PCR mutagenesis such that a single nucleotide deletion was introduced at both nt 2125 and nt 2175 of pCMVNS1/2, shifting the NS2 ORF into the NS1 ORF at nt 2125, then back into the NS2 ORF at nt 2175. pHAUb was constructed by inserting an oligonucleotide coding for the HA tag YPYDVPDYA into pcDNA3.1 and then inserting the ubiquitin gene from pYEP96 (Ecker *et al.,* 1987) in frame behind the HA tag. SP6NS2 was previously described (Lorson *et al.,* 1996). pGEX-2TK-Ubiquitin was a gift from John Cannon, University of Missouri. pCS2p21, myc-tagged pCS2+MTcyclinE, and pUbR7 were gifts from B. Clurman (Sheaff *et al.,* 2000).

Pulse–chase and immunoprecipitation assays

Cells were labeled with 100 mCi/ml ^{35}S 18 h posttransfection or 24 h postinfection and pulse–chase assay was performed as previously described (Schoborg and Pintel, 1991) with the following changes. Proteasome inhibitors MG132 (10 μM), lactacystin (5 μM), and epoxomycin (10 μM) (Affiniti Research Products Limited, Exeter, UK) were added to cells 3 h prior to labeling where indicated and were present throughout the pulse and chase periods. When labeling pCS2p21- or pCS2-MTcyclinE- transfected cells, ^{35}S exposure time was 30 min. When labeling MVMp-infected or pCMVNS1/2 (and derivative plasmids)-transfected cells, ^{35}S exposure time was 60 min. Labeled cells were collected and immunoprecipitation was performed as described (Clemens *et al.,* 1990) with the following changes. Cells were lysed in 2% SDS and further denatured by 10-min incubation at 90°C. RIPA buffer (Naeger *et al.,* 1990) was added to final SDS concentration of 0.25%. Five microliters from each sample was removed and counted by scintillation counter. Equivalent counts from each sample were added to the appropriate antibody for incubation. After immunoprecipitation, dried gels were exposed to a Kodak phosphoimaging screen (Bio-Rad) and quantitated using a Bio-Rad Imager FX and Quantity One software. Where applicable, NS2 counts were equilibrated to NS1 counts to control for sample loading differences. Antibodies used include rabbit polyclonal α-NS2 C-terminal, made against C-terminal NS2 peptide aa 156–182; rabbit polyclonal α-NS1/2 N-terminal, made against a peptide containing aa 5–25 shared by both NS1 and NS2 proteins; rabbit polyclonal α-p21 (C19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and mouse monoclonal α-c-myc (Boehringer Mannheim).

TsA1S9 assay

Assays were done as has been previously described by Clurman *et al.* (1996). Six hours posttransfection of tsA1S9 cells at 32°C, MG132 (10 μM) was added to four of eight transfected plates. Two plates containing MG132, and two plates without MG132, were then shifted to the nonpermissive temperature of 40°C, while two plates containing MG132, and two plates without MG132, remained at the permissive temperature of 32°C. After a 12-h incubation, the cells were collected and lysed, and total protein levels were determined by Bradford assay. Equivalent amounts of protein were run on SDS–PAGE and transferred to nitrocellulose. Western blot analysis was performed as described (Amersham, Inc.) and the proteins were visualized by chemiluminescence (Pierce SuperSignal) on film. In parallel, blots were exposed to a chemiluminescence screen (Bio-Rad) and quantitated using a Bio-Rad Imager FX and Quantity One software.

Immunoprecipitation and Western analysis

MG132 (10 μM) was added to cells where indicated 6 h posttransfection. Cells were collected 18 h posttransfection and lysed in nondenaturing Raf buffer (Bodendorf *et al.,* 1999) containing 1× protease inhibitors (Sigma). Lysates were added to 8 μl antibody and incubated, rotating at 4°C for 4 h. One hundred microliters of a 50:50 Raf buffer:protein A–Sepharose slurry was added, and the samples were incubated for an additional hour. Samples were washed three times with Raf buffer, resuspended in 1× IP dye, run on 10% SDS–PAGE, and transferred to nitrocellulose, and Western blot analysis was performed as described (Amersham, Inc.). Blots were visualized by chemiluminescence on film. Antibody not previously mentioned was mouse monoclonal α-HA.11 (Covance, Richmond, CA).

In vitro GST pull-down assays

GST–ubiquitin was made, purified, and labeled with ^{32}P as previously described (Mouw and Pintel, 1998). ^{35}S-labeled, or unlabeled, TNT-generated NS2 was made as previously described (Lorson *et al.,* 1998). Beaded labeled GST–ubiquitin and cold NS2, or cold beaded GST–ubiquitin and labeled NS2, were incubated together in an S100 cytoplasmic extract or reticulocyte lysate for 15 or 90 min, washed three times, and run on SDS–PAGE. Gels were dried and exposed to film to visualize possible interactions.

Z-vad-FMK inhibitor assay

Eighteen hours posttransfection, A92L cells were treated with caspase inhibitor z-vad-FMK (Enzyme Systems Products, Livermore, CA) at a final concentration of 20 μM, as indicated by the supplier. After a 2-h incubation, pulse–chase analysis and immunoprecipitation were performed as described above.

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